N-POLYMETHYLENECARBOXYMALEIMIDES – A NEW CLASS OF PROBES FOR MEMBRANE SULPHYDRYL GROUPS

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1. Introduction

Many membrane proteins contain essential sulphydryl groups and are therefore susceptible to modification and subsequent inhibition by electrophilic reagents. The availabilty of non-penetrant sulphydryl reagents such as *p*-chloromercuriphenylsulphonate and permeant reagents such as *N*-ethyl maleimide has allowed the localisation of sulphydryl groups at either face of the membrane or within the membrane. Determination of the depth of sulphydryl groups within the membrane has not been possible until recently. Reagents consisting of a hydrophilic spacer arm and a reactive maleimide residue have been used to probe populations of sulphydryl groups on or inside the erythrocyte membrane [1]. Similar reagents have been employed in investigations of the role of sulphydryl groups in the transport of glucose into brush border membrane vesicles [2]. We have synthesised a series of *N*-polymethylenecarboxymaleimides for use as membrane-impermeant sulphydryl reagents. The positions of some of the essential sulphydryl groups of the phosphate-transporter protein of insect flight muscle and rat liver mitochondria have been determined using these compounds.

2. Materials and methods

Compound AM1 (see table 1 for abbreviations) was synthesised according to [13]. Compound AM2 was synthesised using the method in [14]. Compounds AM3 and AM4 were synthesised from the corresponding aminoalkanoic acids by a modification of the method in [3]. AM5, AM7, AM10 and AM11 were synthesised from the maleamic acids as in [4]. Partition coefficients were measured by vortexing 1 ml *n*-octanol with 1 ml potassium phosphate buffer (pH 8.0) containing 1 mM *N*-polymethylenecarboxymaleimide for 30 s. The concentration of the maleimide in each phase was determined by HPLC on a reversed phase-bonded silica column (Lichrosorb RP 2) eluted with methanol/50 mM potassium phosphate, pH 4 (3:2) at 1 ml/min. Maleimides were detected by their absorption at 300 nm.

Insect flight muscle mitochondria were prepared from *Sarcophaga barbata* as in [5]. Rat liver mitochondria were prepared as in [6]. Rat liver mitochondria (3 mg, 50 μl) were diluted with 250 μl of a solution containing 200 mM sucrose, 50 mM KCl, 20 mM Tris–HCl, 1 mM EDTA (pH 8.0) and incubated with the appropriate maleimide solution (20 nmol/mg protein) for 1 min at 22°C. The reaction was terminated by the addition of β-mercaptoethanol to 0.5 mM. Aliquots of 1 ml were removed for the determination of phosphate transport as in [7]. Insect flight muscle mitochondria were incubated with maleimides (20 nmol/mg protein) in a medium containing 150 mM KCl, 10 mM sodium phosphate, 2 mM EGTA and 1 mM $CaCl_2$ (pH 7.4). The reaction was terminated with β-mercaptoethanol and the ability to transport phosphate was assayed as above.

3. Results and discussion

The chainlength and the partition coefficients of the *N*-polymethylenecarboxymaleimides used are listed in table 1. The introduction of extra methylene groups causes an increase in the overall hydrophobicity as measured by the partition coefficient of the compounds between *n*-octanol and phosphate buffer (pH

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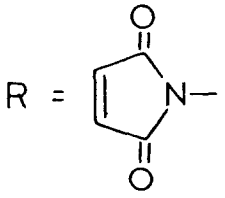
Table 1
Properties of *N*-polymethylenecarboxymaleimides

Compound	Abbreviation	Partition coefficient	Distance[a] (Å)
R = 			
R. CH_2COOH	AM1	0.03	5.9
R. $(CH_2)_2COOH$	AM2	0.03	7.1
R. $(CH_2)_3COOH$	AM3	0.03	8.3
R. $(CH_2)_4COOH$	AM4	0.03	9.6
R. $(CH_2)_5COOH$	AM5	0.05	10.8
R. $(CH_2)_7COOH$	AM7	0.09	13.2
R. $(CH_2)_{10}COOH$	AM10	0.34	17.1
R. $(CH_2)_{11}COOH$	AM11	2.84	18.3
CH_2CH_3	NEM	3.0[b]	–

[a] The distance is that measured from the reactive maleimide moiety to the carbon atom of the carboxyl group; [b] Data from [8]

8.0). However, even AM10 is relatively water-soluble compared to NEM, indicating that the carboxyl group is well ionised under the conditions of the assay and hence will not penetrate the membrane. The structure of the compounds is such that the molecules should orientate perpendicular to the plane of the membrane, as found for other membrane probes with analogous structures such as the *n*(9-anthroyloxy)-fatty acids [10], or the *N*-oxyl-4′,4′-dimethyloxazoladine derivatives of ketostearic acids [9]. The varying lengths of the hydrophobic spacer arms permit reaction with sulphydryl groups at varying depths within the lipid bilayer.

The effects of these compounds on the transport of phosphate across the inner mitochondrial membrane was studied using the energised swelling technique [7]. Under the conditions of this assay system, in the presence of an uncoupling agent and ATP, the inhibition of phosphate transport leads to an increase in the intramitochondrial content of phosphate and a consequent osmotically induced swelling of the mitochondria. The phosphate transporter of rat liver mitochondria has been shown to be inhibited by mercurials and maleimides [11,12]. Hence the ability of *N*-polymethylenecarboxymaleimides to induce swelling was investigated. The results are presented in fig.1.

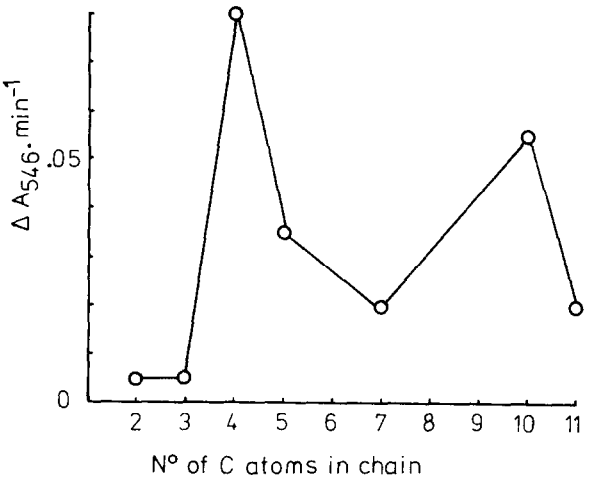


Fig.1. The effects of *N*-polymethylenecarboxymaleimides on phosphate transport in rat liver mitochondria. Incubation of mitochondria with the maleimides was as in [7]. Swelling was measured by following the increase in absorbance at 546 nm.

It can be seen that the rate of swelling induced by incubation with the different chain length *N*-polymethylenecarboxymaleimides varies with the length of the polymethylene side chain. The fastest rate of swelling was observed with AM4 and AM10. This rate of swelling is comparable to that induced by NEM, a permeant sulphydryl reagent. There is a large difference in the abilities of AM3 and AM4 to induce swelling. AM7 and AM10 are relatively ineffective at inducing swelling. These results do not correlate with the measured partition coefficients, (hydrophobicity) of these compounds (see table 1) and hence cannot be explained solely in terms of their solubility in the lipid bilayer.

The swelling of insect flight muscle mitochondria after treatment with the *N*-polymethylenecarboxymaleimides is shown in fig.2. In this case swelling comparable to that induced by NEM is found after incubation with AM3 and AM10. Compounds AM1, AM2 and AM11 are relatively ineffective, whilst compounds AM4, AM5 and AM7 induce an intermediate rate of swelling.

We conclude that these results indicate that there are 2 populations of sulphydryl groups which are involved in the process of the ATP-induced swelling of mitochondria. These exist at different levels in the plane of the mitochondrial membrane. They are probably involved with the phosphate transporter protein since control experiments have shown that the other

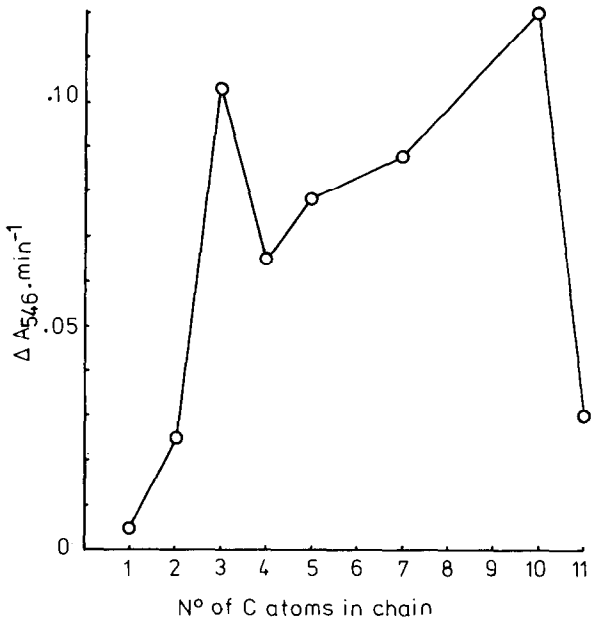


Fig.2. The effects of *N*-polymethylenecarboxymaleimides on phosphate transport in insect flight muscle mitochondria. Conditions for incubation were as in section 2. Swelling was monitored by following the increase in absorbance at 546 nm.

enzymes involved in this assay system, i.e., the adenine nucleotide translocator and the proton-ATPase are not affected significantly by treatment with these compounds under the conditions described here. It is possible that these 2 populations represent the same sulphydryl group in 2 environments caused by conformational changes in the structure of the phosphate transporter. The speed of the reaction of the sulphydryl group with the maleimides at different depths in the membrane could reflect the relative proportion of time spent by the sulphydryl group at these depths. If this hypothesis is correct then the relative inefficiency of the short chain *N*-polymethylenecarboxymaleimides in inducing swelling would indicate that the sulphydryl group is always at a depth >8 Å into the membrane (see table 1). The high reactivity of AM4 and AM10 (for rat liver mitochondria) or AM3 and AM10 (for insect flight muscle mitochondria) could represent 2 preferred conformations of the sulphydryl group. The intermediate swelling induced by AM5, AM7 and AM11 could represent the relatively shorter time spent in the transition from one preferred conformation to another. This model assumes that the reactivity of the sulphydryl group remains constant at all depths in the membrane.

An alternative model is that the 2 populations of sulphydryl groups represent discreet cysteine residues on the phosphate transporter protein. The intermediate reactivities of AM5, AM7 and AM11 could then relate to a rocking movement of the phosphate transporter in the membrane causing the sulphydryl groups to be accessible to the maleimides for relatively short periods.

The mechanisms whereby membrane proteins carry out vectorial processes such as transport are almost unknown; only recently was the topology of these processes investigated. These impermeant maleimides should prove valuable in such studies. We are synthesising ranges of other impermeant compounds which react with different functional groups in proteins in order to probe other residues associated with membrane-associated phenomena.

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